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Long lasting immunity in trichinellosis – insight from a small study group

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Abstract

Trichinellosis in humans is most often caused by the parasite *Trichinella spiralis*. The clinical course of the disease is diverse and the symptoms can vary from mild to severe. Symptoms usually disappear within a few months, but encysted larvae in the muscles can cause myalgia and weakness that last for years. However, the existence of chronic trichinellosis as a disease is still debatable. This study presents the results obtained at the National Reference Laboratory for Trichinellosis – INEP, Serbia. The study was conducted to assess the immunoserological, biochemical and symptomatic disease parameters of twelve patients who acquired trichinellosis 13 and 18 years ago, respectively. They were involved in two *T. spiralis* outbreaks in Serbia, at the village of Kumane and the city of Belgrade (nine and three patients, respectively). Results indicated the presence of specific anti-*Trichinella* antibodies in 83% of the total number of patients. However, while the humoral immune response to *Trichinella* lasted for more than a decade reaching almost two decades after the acute infection phase (7/9 and 3/3 respectively, in two outbreaks), persistence of chronic muscular pain, as the most prolonged symptom of trichinellosis, could be found in the majority of patients from the Kumane outbreak (7/9). As a consequence, these patients suffered from limitations in daily living activities for the same period of time. The results presented in this paper are our contribution to the view that trichinellosis as a chronic disease with symptoms exists and may be related to the severity of the disease in the acute phase.

Introduction

Trichinellosis is a parasitic disease in humans caused by the nematodes of the genus *Trichinella*. Infection can be divided in intestinal (enteral) phase and systemic (parenteral) phase (Gottstein *et al.*, 2009). In the enteral phase, infectious *Trichinella* larvae develop into adults, and in the parenteral phase, newborn larvae migrate to the striated muscles (Dupouy-Camet & Murrell, 2007). In most cases, trichinellosis resolves without complications within several months. However, in some patients the chronic muscular pain persists for years, which supports the existence of a chronic phase of the disease (Harms *et al.*, 1993). A number of researches have considered a chronic form of trichinellosis to be the perennial presence of symptoms such as myalgia, fatigue, ocular signs and headache, while others prefer the term 'persisiting sequelae' (Bruschi & Murrell, 2002). Clinical manifestations could result from the presence of *Trichinella* larvae in skeletal muscle, where it can survive up to 40 years (Fröscher *et al.*, 1988), but the mere larvae existence and vitality in the muscles does not necessarily mean that a person will develop symptoms (Dupouy-Camet *et al.*, 2002). What it certainly means is that live muscle larvae continuously release metabolic products (excretory–secretory antigens, ES L1) into the circulation, which induce the production of anti-*Trichinella* antibodies. Differences in immunoglobulin classes that can be detected during infection depend on the time of seroconversion (van Knapen *et al.*, 1982). The time of seroconversion is affected by the number of ingested larvae, the *Trichinella* species involved and the host's immune response (Pozio *et al.*, 1993). The level of specific immunoglobulin E (IgE), immunoglobulin G (IgG) and immunoglobulin G4 (IgG4) antibodies against *Trichinella* spp., as well as levels of creatine phosphokinase (CK) and lactate dehydrogenase (LDH) as non-specific laboratory findings indicating muscle damage, generally increase in the parenteral phase of trichinellosis (Bruschi & Murrell, 2002). Like other helminth infections, *Trichinella* infection induces the specific IgE humoral immune response. IgE antibodies, provoked by *Trichinella* infection, appear early and are typical for the acute stage of the disease (Gottstein *et al.*, 2009). These antibodies play a role in allergy-like symptoms typical of trichinellosis, such as periorbital oedema and cutaneous rash (Watanabe *et al.*, 2005). Anti-*Trichinella* IgG antibodies can be detected in serum 12 to 60 days after infection and may persist in the blood for years (Harms *et al.*, 1993; Gottstein *et al.*, 2009). Inspired by

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Table 1. Epidemiological and clinical characteristics of patients from the 1997 and 2002 *Trichinella* outbreaks.

Year of outbreak and location	Year of blood sampling	Number of years since *Trichinella* infection	Patient number	Age	Gender	Source of infection	Clinical course of acute trichinellosis	Days in hospital during outbreak	Symptoms of the presence of chronic trichinellosis
1997 Belgrade	2015	18	1	48	Male	Sausages, smoked pork	Mild	0	/
			2	50	Male				
			3	45	Male				
2002 Kumane	2015	13	1	56	Male	Sausages, smoked pork	Severe	30	Chronic muscle pain
			2	35	Female		Severe	15	Chronic muscle pain
			3	50	Male		Severe	15	Chronic muscle pain
			4	61	Male		Severe	10	Chronic muscle pain
			5	42	Male		Severe	17	Chronic muscle pain
			6	40	Female		Severe	10	/
			7	38	Male		Moderate	3	/
			8	39	Male		Severe	10	Chronic muscle pain
			9	57	Female		Mild	0	Chronic muscle pain

the question of the existence of chronic trichinellosis, in 2015 we conducted a study to assess the humoral immune response of patients from two outbreaks in Serbia that occurred 13 and 18 years ago and to correlate it with biochemical parameters and symptoms that the subjects have been complaining about continuously since the time of *Trichinella* infection. The persistence of specific IgG and IgG4 antibodies, elevated levels of LDH and chronic muscular pain existence were found in a significant number of *Trichinella spiralis* infected patients included in this study and are described in this paper.

Materials and methods

Patients

There were 12 individuals who gave informed consent to participate in the study. Three persons belonged to the group of 15 patients involved in an outbreak reported in the capital city of Belgrade, Serbia in 1997. Nine study participants were among 309 patients from a large outbreak registered in the village of Kumane, municipality of Zrenjanin, Central Banat District in Serbia on 30 December 2001 and in the first half of January 2002. Infection in both outbreaks was caused by consumption of smoked sausages prepared from uninspected pork containing *T. spiralis* larvae. In order to assess patients' perception of their current health status and to estimate the prevalence of reported symptoms, they filled in the health questionnaire. Retrospective data regarding the course of acute trichinellosis and treatment were collected from the patients' medical history.

Laboratory testing

Collection of serum samples was performed in the Institute for the Application of Nuclear Energy – INEP and Primary Health Center Novi Becej in March 2015. All the sera were tested for anti-*Trichinella* antibodies by indirect immunofluorescence antibody assay IFA ('FITC *Trichinella spiralis* Antibody Detection Kit', INEP, Serbia). For detection of *Trichinella*-specific IgG, IgG4 and IgE antibodies in patients' sera, the in-house indirect enzyme-linked immunosorbent assay (i-ELISA) tests were used (Devic *et al.*, 2014; Gnjatovic *et al.*, 2019). The diagnostic specificity of the in-house IgG i-ELISA test was 97.5% and the sensitivity 87.5%, while the IgG4 i-ELISA had a specificity of 67.5% and a sensitivity of 97.5%, and IgE i-ELISA 92.9% and 100%. Biochemical analysis (performed at INEP, Department for Metabolism) included determination of muscle enzymes: CK and LDH.

Statistical analyses

GraphPad Prism 8.0 (GraphPad Software, California, USA) was used for analysing data and drawing charts. The data are expressed as the mean ± standard deviation. Correlation analysis was performed to determine the relationship between serum IgG and IgG4 levels, as well as CK and LDH. Pearson correlation coefficients (r) and corresponding P values were determined using X–Y correlation analysis. A P value less than 0.05 was considered statistically significant.

Results

Patients

Persons included in this study were adults ($n = 12$), nine males with a median age of 48 years (range 38–61) and three women with a median age of 44 years (range 35–57) (table 1). They all met the case definition criteria for trichinellosis, for example, they had at least three out of six defined clinical signs and

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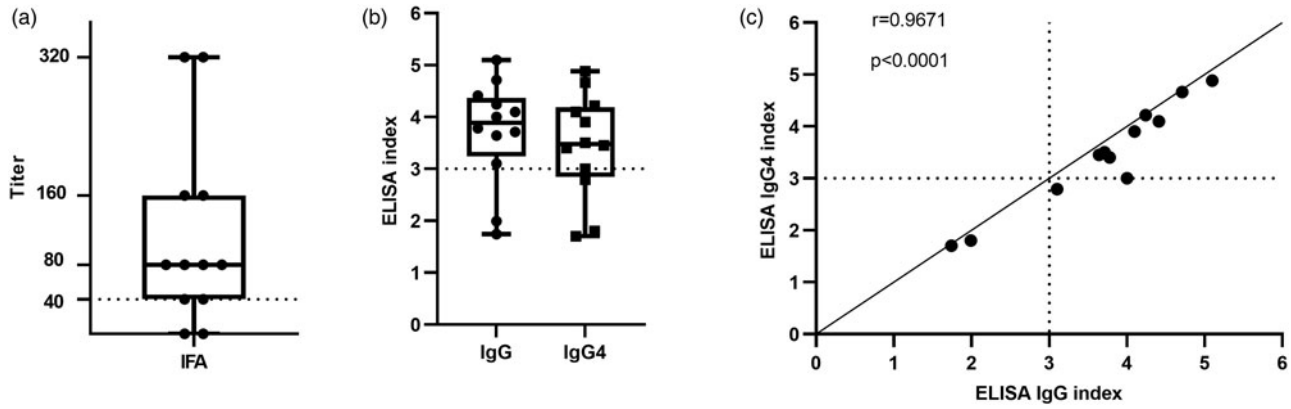

Fig. 1. Summary of immunofluorescence antibody assay (IFA) and enzyme-linked immunosorbent assay (ELISA) test results of 12 patients from the 1997 and 2002 *Trichinella* outbreaks. (a) IFA titre. (b) ELISA immunoglobulin G (IgG) and immunoglobulin G4 (IgG4) index. (c) Correlation between ELISA IgG and IgG4. Boxes represent the interquartile range and horizontal lines inside each box represent the median. The vertical lines from the ends of each box encompass the extreme data points. The levels of IgG and IgG4 were significantly correlated ($P < 0.0001$).

symptoms at the onset of the disease (Dupouy-Camet & Murrell, 2007) and were positive for the presence of *Trichinella*-specific antibodies. Seven study participants had severe symptoms during acute infection and were hospitalized (all from the 2002 outbreak), one had a moderate course of infection and was also hospitalized (from the 2002 outbreak) and four had a mild form of the disease (one from the 2002 outbreak, three from the 1997 outbreak) (table 1). Hospitalized patients were treated with mebendazole and corticosteroids, while the others were ambulatory treated with mebendazole. Data obtained by questionnaire indicated that after 13 years from acute infection seven patients, all from the 2002 outbreak, complained to have chronic myalgia periodically, while the rest of them denied existence of any clinical signs regarding trichinellosis (table 1).

Laboratory testing

The presence of anti-*Trichinella* antibodies in the sera of study participants was first examined by IFA, and 10 out of 12 subjects were positive for anti-*Trichinella* antibodies (cut off value 1:40). So, 83% of study participants tested positive with an IFA titre ranging from 1:40 to 1:320 with a mean titre of 136 ± 105.32. The mean titre in the sera of the 1997 outbreak participants was 266.67 ± 92.38, whereas the mean titre detected in the 2002 outbreak participants was 62.22 ± 49.44.

Testing of sera samples with anti-*Trichinella* IgG ELISA revealed that 83% (10/12) of study participants were anti-*Trichinella* IgG positive, while the results of anti-*Trichinella* IgG4 ELISA indicated that 75% (9/12) subjects were IgG4 positive. The ELISA index cut off value was 3.00. Mean serum levels of IgG and IgG4 expressed through index value were 3.71 ± 1.01 and 3.45 ± 1.01, respectively. In the sera of the study participants from the 1997 outbreak, mean serum IgG and IgG4 levels were 4.68 ± 0.43 and 4.59 ± 0.34, respectively, while these levels in the sera from the 2002 outbreak participants were 3.39 ± 0.94 and 3.07 ± 0.85, respectively. Next, we analysed whether serum levels of IgG were correlated with IgG4 in all patients and we found that these levels correlated significantly (Pearson's correlation coefficient $r = 0.9671$, $P < 0.0001$). IgE immunoglobulin was not detectable in all sera samples. Serological results are shown in fig. 1 and Supplementary table S1.

Biochemical analyses of CK and LDH in serum samples showed that 58% (7/12) of patients still have elevated LDH (ref. range <227 U/L) with the mean level 375.16 ± 82.06. Elevated CK level (ref. range <200 U/L) with value of 265 was detected in only one patient (from the 2002 outbreak), who also had raised LDH. Mean serum levels of CK and LDH in the sera from the 1997 outbreak patients were 83 ± 44.17 and 113.37 ± 34.86, respectively, while these levels in the sera from the 2002 outbreak patients were 92 ± 81.53 and 324.13 ± 124.90, respectively. We then analysed whether serum LDH levels correlated with CK in all study participants, but as expected, there was no statistical significance. Results are shown in fig. 2 and Supplementary table S2.

Discussion

In this study, laboratory findings showed that the humoral immune response to *Trichinella* still persists 18 and 13 years after acute infection. Re-evaluation of three patients from the 1997 (Belgrade) outbreak has shown that they still have anti-*Trichinella* IgG and IgG4 antibodies. Biochemical analysis revealed CK and LDH levels within normal limits, and the questionnaire showed that none of them complained of any symptom characteristic for trichinellosis. On the other hand, among nine patients from the Kumane outbreak in 2002, 78% (7/9) of them had chronic muscular pain symptoms. Five of them had increased both IgG and IgG4 antibodies, one had increased IgG antibodies with the IgG4 subclass in the normal range, while one patient had no anti-*Trichinella* antibodies. From the two persons who did not complain of symptoms of the muscular system disturbances, one had anti-*Trichinella* antibodies.

Our results are in line with the study of Kociecka *et al.* (2001), which demonstrated persistence of chronic trichinellosis signs and symptoms in 88.7% of 44 patients, one to seven years after acute trichinellosis, while 86.4% had a positive ELISA test for antibodies against excretory–secretory antigens of *Trichinella* sp. The results presented here are also in agreement with the observations from another study that antibodies to *T. spiralis* were still detectable 10 years after infection in more than one-third of patients in a follow-up investigation of 128 patients (Harms *et al.*, 1993). Anti-*Trichinella* antibodies (IgG, IgG4) were also found in patients 15 years after *Trichinella britovi* infection (Piergili-Fioretti *et al.*, 2005; Pinelli *et al.*, 2007). The persistence of antibodies against *Trichinella* can be explained by the constant

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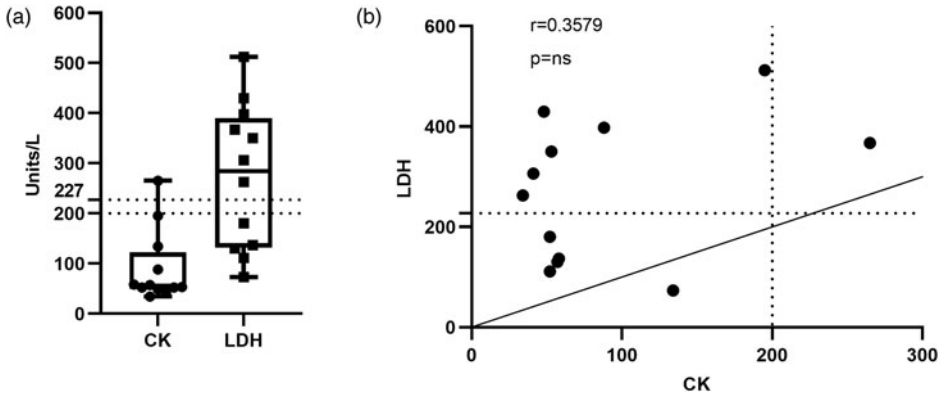

Fig. 2. Summary of creatine phosphokinase (CK) and lactate dehydrogenase (LDH) analysis of 12 patients from the 1997 and 2002 *Trichinella* outbreaks. (a) CK and LDH level. (b) Correlation between CK and LDH. Boxes represent the interquartile range and horizontal lines inside each box represent the median. The vertical lines from the ends of each box encompass the extreme data points. ns = not significant.

release of ES L1 antigens from the muscle larvae of the parasite that induces synthesis of different subclasses of specific immunoglobulins. The results of the ELISA test for *Trichinella*-specific IgG4 antibodies in the sera of patients from these two outbreaks indicate a high level of agreement with those obtained for IgG-specific antibodies. We could conclude, based on a relatively small number of analysed sera that specific IgG antibodies present in the late period of *Trichinella* infection are predominantly of IgG4 nature. It was previously noted that in the chronic stage of infection continuous stimulation with muscle larvae ES L1 antigens predominantly induces secretion of IgG4 and that in endemic areas its presence could be associated with reinfection (Ljungström *et al.*, 1988; Pinelli *et al.*, 2004). Follow-up of 15 patients for one year after infection showed significant increase in the IgG4 antibody level in the sera samples during the chronic phase compared to the early phase of infection (Ljungström *et al.*, 1988). However, Pinelli *et al.* (2001, 2004, 2007) observed that the IgG4 immune response could be detected during both early and chronic stages of trichinellosis. As we have already emphasized, IgE antibodies are typical for the early phase of the disease (Gottstein *et al.*, 2009). Since we did not detect IgE antibodies in examined sera we can assume that the existence of anti-*Trichinella* IgG4 antibodies is not the consequence of reinfection. In addition, it was found that the levels of anti-*Trichinella* antibodies in examined sera do not depend on the time that had passed since the infection, because the level of antibodies was higher in the sera from the 1997 outbreak. Also, all patients from the 1997 outbreak had a mild form of trichinellosis, so higher levels of antibodies in the chronic phase of trichinellosis could not be associated with the previously observed severity of the acute form of the disease.

Biochemical parameters indicating possible muscle damage, such as muscle enzymes LDH and CK, were also determined. LDH is a non-specific marker of tissue damage, while raised values of CK were associated with skeletal muscle destruction caused by the invasion of *Trichinella* larvae (Capo & Despommier, 1996). Biochemical analysis of serum samples showed that even after many years, seven out of nine patients still had elevated LDH levels, and only one of them also had elevated CK levels. These results are in line with results from the literature, which described that in 13 out of 48 patients from Italy, 15 years after the initial *T. britovi* infection, one patient had an elevated level of CK and two patients had elevated levels of LDH (Piergili-Fioretti *et al.*, 2005). Seven patients (58%) from our study complained of muscular pain during the chronic phase of trichinellosis. Six of them were hospitalized with an acute infection which demonstrated severe clinical signs, and one patient had a mild course of disease, which could mean that the risk of developing chronic trichinellosis may be related to the severity of the disease in the acute phase. The severity of trichinellosis depends on the number of ingested living larvae on one side and gender, age, immune status of host on the other (Bruschi & Murrell, 2002). Muscle pain that is experienced during acute trichinellosis may be the consequence of the damage of skeletal muscle caused by nurse cell complex formation (Kociecka, 2000). Based on the anamnestic data on the presence of myalgia, we can only hypothesize that these patients, who have been infected a long time ago, may possess the large number of larvae that are still alive in the muscle tissue. The complex mechanisms by which larvae act on the neuroendocrine–immune axis have not yet been elucidated, but it could be assumed that molecular crosstalk between host and parasite underlies the occurrence and duration of muscle pain symptoms in chronic trichinellosis. In this study, chronic myalgia could not be correlated with the level of muscle enzymes LDH and CK, whose increase in serum indicates muscle tissue damage. The connection between the existence of live larvae, the appearance of antibodies and the symptoms of muscle pain was also indicated by the results of other authors. From the study which covered a period from 1.5 to 42 years after infection it could be seen that there was antibody presence along with the existence of live *Trichinella* larvae and focal myositis in muscles, accompanied by myalgia or without it (Fröscher *et al.*, 1988). Follow-up of 699 patients diagnosed with trichinellosis over a two-year period in Romania indicated that myalgia and fatigue could last in some cases for up to 24 months (Nemet *et al.*, 2009). Also, symptoms of impaired muscle strength have been reported in patients 10 years post-infection (Harms *et al.*, 1993). Autoimmune response triggered against skeletal muscle antigens could also be the reason for muscle damage. The presence of specific anti-skeletal muscle antibodies that recognize 27 and 41 kDa proteins in muscle tissue extract has been described in patients who had *T. spiralis* infection 3–8 years earlier (Pratesi *et al.*, 2006). Additionally, in the study of 20 patients with acute trichinellosis confirmed by anti-*Trichinella* antibodies, all sera showed the presence of anti-striational antibodies which may suggest the occurrence of an anti-muscle autoimmune response (Macura-Biegun *et al.*, 1998). Cross-reactivity between host tissue and *Trichinella* antigens may be the reason for induced autoimmune response (Radovic *et al.*, 2012).

An interesting observation emerged from this study that three participants noticed some improvement in their health condition during the years after *Trichinella* infection. Two of them emphasized that for many years they did not have even influenza, while one patient declared no relapses of labial herpes simplex infection. Experimental studies have suggested that the regulatory immune

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response induced by helminths could control excess inflammation and hence participate in the modulation of inflammatory diseases, such as allergies and autoimmune diseases (Aranzamendi *et al.*, 2013; Maizels, 2020). Double-blind clinical trials in humans, designed to use helminths as therapeutic agents for allergic diseases have not shown significant improvements, but the use of the parasites in the treatment of autoimmune disorders has reduced symptoms of multiple sclerosis, ulcerative colitis and Crohn's disease (Ryan *et al.*, 2020). Here, the presented laboratory results indicate continuous antigenic stimulation by *Trichinella* for a long period of time, more than a decade after infection, so it is possible that immune regulatory responses induced by this parasite may be the reason for better health status. Although subjective, such statements about the improvement of health condition draw attention to the need for further observations and studies.

The findings in our study had some limitations that should be discussed. Since patients were recruited more than 10 years after the acute infection, the number of those who agreed to participate in the study was limited. The questionnaire used in our research had a limitation reflected in the subjectivity of the respondents' answers, thus the existence of chronic pain in patients remained unverified in terms of the presence of *Trichinella* muscle larvae, due to the fact that muscle biopsy is an invasive and painful procedure for patients. If the option of the mentioned diagnostic method was proposed, even fewer persons would have accepted to participate in our study. To avoid further limitations to our study, we presented the obtained results collectively for both outbreaks, that is, for the total number of patients. Despite all limitations, this study nevertheless demonstrated serological evidence of the presence of anti-*Trichinella* antibodies in these patients. The selected diagnostic methods were appropriate, and the data on which we based our conclusions were valid. The diagnostic results of the IFA test were consistent with the ELISA test and we confirmed that the patients had an immune response against *Trichinella* that lasted for years. This study provided an insight into the persistent immune response associated with *T. spiralis* infection and may guide future investigations on a larger number of patients to determine with certainty whether chronic trichinellosis really exists.

Based on the results presented here, we can conclude that humoral immunity in trichinellosis may last for time, most likely as long as the larvae in the muscles remain alive. The results also indicate that the presence of a moderate or severe clinical picture in the acute phase of the infection could be related to the persistence of trichinellosis symptoms, such as chronic muscle pain, more than a decade after infection. Our opinion is that the existence of chronic trichinellosis is a fact that should not be doubted.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X22000268.

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients prior to collection and analysis of serum samples.

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